Cellular and Molecular Life Sciences (2021) 78:7635–7648
https://doi.org/10.1007/s00018-021-03976-7

ORIGINAL ARTICLE

Sam68 contributes to intestinal inflammation in experimental and human colitis

Wendy A. Goodman[1] · Shrikanth C. Basavarajappa[1] · Angela R. Liu[1] · Franklin D. Staback Rodriguez[1] · Tailor Mathes[1] · Parameswaran Ramakrishnan[1,2,3]

Received: 22 April 2021 / Revised: 29 September 2021 / Accepted: 9 October 2021 / Published online: 24 October 2021

Abstract

Sam68 is an RNA-binding protein with an adaptor role in signal transduction. Our previous work identified critical proinflammatory and apoptotic functions for Sam68, downstream of the TNF/TNFR1 and TLR2/3/4 pathways. Recent studies have shown elevated Sam68 in inflamed tissues from rheumatoid arthritis and ulcerative colitis (UC) patients, suggesting that Sam68 contributes to chronic inflammatory diseases. Here, we hypothesized that deletion of Sam68 is protective against experimental colitis in vivo, via reductions in TNF-associated inflammatory signaling. We used Sam68 knockout (KO) mice to study the role of Sam68 in experimental colitis, including its contributions to TNF-induced inflammatory gene expression in three-dimensional intestinal organoid cultures. We also studied the expression of Sam68 and inflammatory genes in colon tissues of UC patients. Sam68 KO mice treated with an acute course of DSS exhibited significantly less weight loss and histopathological inflammation compared to wild-type controls, suggesting that Sam68 contributes to experimental colitis. Bone marrow transplants showed no pathologic role for hematopoietic cell-specific Sam68, suggesting that non-hematopoietic Sam68 drives intestinal inflammation. Gene expression analyses showed that Sam68 deficiency reduced the expression of proinflammatory genes in colon tissues from DSS-treated mice, as well as TNF-treated three-dimensional colonic organoids. We also found that inflammatory genes, such as *TNF, CCR2, CSF2, IL33 and CXCL10*, as well as Sam68 protein, were upregulated in inflamed colon tissues of UC patients. This report identifies Sam68 as an important inflammatory driver in response to intestinal epithelial damage, suggesting that targeting Sam68 may hold promise to treat UC patients.

Keywords KHDRBS1 · Inflammatory bowel disease · Inflammation · NF-kappaB · TNF

Introduction

Ulcerative colitis (UC) is the most prevalent form of inflammatory bowel disease (IBD) in adults, affecting more than one million people in the USA (Crohn's disease Factbook 2019) and increasing in prevalence worldwide. UC is idiopathic and involves a complex interplay of genetic, environmental and immune components that result in chronic, diffuse inflammation of the colon and rectum [1]. Chronic inflammation in the intestinal mucosa of UC patients leads to a relapsing and remitting disease course that is characterized by bloody diarrhea, abdominal pain and cramping, cachexia, and anemia. Patients with UC are at significantly increased risk for the development of colorectal cancer and extra-intestinal manifestations including primary sclerosing cholangitis and urolithiasis [2].

Recent therapeutic advances, including the use of targeted biologics, have resulted in improved quality of life and reduced mortality for UC patients [3]. However, mucosal healing does not always accompany symptom control [4], and patients often experience unwanted and/or potentially-severe side effects [5]. Therefore, an improved understanding of the molecular events regulating inflammatory signaling

✉ Parameswaran Ramakrishnan
pxr150@case.edu

[1] Department of Pathology, School of Medicine, Case Western Reserve University and University Hospitals Cleveland Medical Center, 2103 Cornell Road, Room 6526, Wolstein Research Building, Cleveland, OH 44106, USA

[2] Department of Biochemistry, School of Medicine, Case Western Reserve University, Cleveland, OH 44106, USA

[3] The Case Comprehensive Cancer Center, School of Medicine, Case Western Reserve University, Cleveland, OH 44106, USA

pathways in UC is critical for the development of safer, more efficacious therapeutics.

The inflamed intestinal mucosa of UC patients exhibits hyper-activation of several proinflammatory signaling cascades, including tumor necrosis factor (TNF) and toll-like receptors (TLR) [6–8]. TNF is a key regulator of both acute and chronic inflammation [9, 10]. TLRs contribute to inflammation through recognition of conserved molecular patterns associated with gut microbes that translocate through damaged intestinal epithelial cells (IEC), leading to chronic immune activation in the UC mucosa [7]. In previous work, we discovered that Src Associated substrate in Mitosis of 68 kDa (Sam68, also known as KH domain-containing, RNA-binding, signal transduction-associated protein 1 (KHDRBS1)), a member of the signal transduction and activation of RNA metabolism (STAR) family of proteins [11], is required for both TNF [12]- and TLR [13]-mediated proinflammatory signaling. These findings place Sam68 at the nexus of multiple inflammatory signaling cascades with established roles in the pathogenesis of UC.

Sam68 is a ubiquitously expressed, bona fide RNA-binding protein with most of its known functions associated with RNA metabolism, including transcription, alternative splicing and nuclear export [11]. Structurally, Sam68 contains six proline-rich regions (PRRs) with potential to bind –SH3 domain-containing proteins, and a tyrosine phosphorylation region to bind –SH2 domain-containing proteins [11]. Through its PRRs, Sam68 binds to –SH3 domains of SRC kinases and tyrosine phosphorylated Sam68 binds to –SH2 domain containing proteins [14, 15]. Tyrosine phosphorylation of Sam68 by src-like kinases has been shown to counteract its RNA binding and splicing function [16].

Sam68 has been identified as an important regulator of cell survival following DNA damage; in response to genotoxic stress, Sam68 regulates the transactivation of NF-κB-dependent anti-apoptotic genes [17]. Although these functions of Sam68 are protective in the context of cancer treatments, such as γ-irradiation and chemotherapeutics, increased expression of Sam68 is also associated with neoplastic transformation and tumor progression in several human cancers [15], including colonic adenocarcinomas [17]. In addition to its role in cancer initiation and progression, Sam68 was recently implicated in chronic auto-inflammatory diseases such as UC [18] and rheumatoid arthritis [19]. In UC patient samples, overexpression of Sam68 was correlated with increased apoptosis (active caspase 3 and cleaved PARP) and activation of proinflammatory NF-κB signaling in intestinal epithelial cells (IECs) [18], suggesting that Sam68 contributes to UC pathogenesis by enhancing NF-κB-dependent inflammation and antagonizing normal wound repair processes, ultimately leading to apoptosis in IECs. This is distinct from its role in cancer, in which Sam68 expression leads to transactivation of anti-apoptotic genes and increased cell survival [17]. Collectively, these observations suggest that Sam68's complex signaling—including multiple post-translational modification sites and interaction domains—confers multi-functionality and diverse effects on inflammation.

In the present study, we examined the impact of Sam68 deletion on acute, experimental colitis in vivo, testing the hypothesis that mice lacking global expression of Sam68 (Sam68 KO) will be protected from dextran sulfate sodium (DSS)-induced colitis via restricted activation of TNF-dependent NF-κB signaling. Indeed, we observed significant protection among Sam68 KO mice fed 3% DSS for 5 days, compared to wild-type (WT) littermate controls. Bone marrow transplant experiments revealed that hematopoietic cell-specific Sam68 did not contribute significantly to experimental colitis, suggesting that Sam68 in the epithelial compartment (IECs) is the primary driver of mucosal inflammation in response to DSS. To delineate the mechanism(s) underlying decreased inflammation in Sam68 deficient intestine, we examined the expression of selected TNF-associated proinflammatory genes *Tnf*, *Ccr2*, *Csf2*, *Il33*, and *Cxcl10* (encoding TNFα, CCR2, GM-CSF, IL-33, and IP-10, respectively), and found their decreased expression in DSS-treated intestinal tissue. Further implicating IEC-specific Sam68's contribution to experimental colitis, we found striking reductions in TNF-induced inflammatory gene expression in three-dimensional intestinal organoids derived from Sam68 KO colonic crypts compared to those derived from WT mice. Consistent with this, inflamed colon tissues from UC patients showed increased expression of proinflammatory genes as well as Sam68 protein levels.

Collectively, our results identify Sam68 as an important enhancer of TNF-mediated inflammation in human and experimental IBD. We propose that elevated levels of Sam68 present in mucosal tissues of UC patients serves to propagate TNF-dependent inflammatory signaling, contributing to unresolved inflammation and intestinal damage. Our findings suggest that Sam68 may represent a novel therapeutic candidate for UC patients.

Materials and methods

Cells

Control and Sam68 knockout mouse embryonic fibroblasts (MEF), Human Embryonic Kidney 293 T cells (HEK293T) and human colon adenocarcinoma cells (HT-29) were grown in DMEM media supplemented with 100 U/ml penicillin/streptomycin, 4 mM L-glutamine and 10% fetal bovine serum.

Human tissue samples

Human colon biopsy samples were obtained through collaboration with the Biorepository Core of the Cleveland Digestive Diseases Research Core Center (DDRCC). No human subjects were recruited for this study. All the biopsy samples from UC patients and healthy controls were slated to be discarded tissues collected in the past, which were then deidentified and stored at the Biorepository Core Facility. These stored tissues were obtained for experiments from the core with no associated patient identifiable information. According to the minimal information provided by the Biorepository Core, UC tissues collected were obtained from areas of active inflammation or from adjacent, uninflamed areas, colon samples from healthy control individuals were obtained during routine colonoscopy procedures, and all patients (UC and control) were ≥ 18 years of age and were not taking biologic therapeutics at the time of sample collection. All studies involving deidentified human samples were approved by the Institutional Review Board of University Hospitals Cleveland Medical Center (Cleveland, OH) as non-human subject research as defined under NIH guidelines 45 CFR46 or 21 CFR56 and HIPAA exempt.

Mice

All procedures involving experimental mice were approved by the Case Western Reserve University Institutional Animal Care and Use Committee. Sam68 KO mice were kindly provided by Dr. Stephane Richard, McGill University, and were propagated on a C57BL/6J background at Case Western Reserve University. Co-housed, wild-type (WT) littermates were used as controls for all experiments. Mice were housed under specific pathogen-free (SPF) conditions, fed standard laboratory chow (Harlan Teklad, Indianapolis, IN), and kept on a 12-h light/dark cycle.

Dextran sulfate sodium (DSS)-induced colitis

Acute experimental colitis was induced in 8- to 12-week-old Sam68 KO and WT littermate mice with 3% DSS (molecular weight, 36,000–50,000 daltons; MP Biomedicals, Solon, OH) dissolved in sterile drinking water given ad libitum for 5 days, followed by 1 day of sterile drinking water without DSS. Mice were killed on day 6.

Histologic assessment of colonic inflammation

Full-length colon tissue was harvested from experimental mice, flushed of fecal contents using PBS, filleted longitudinally, and fixed overnight in Bouin's solution. Ethanol-rinsed tissues were paraffin embedded, cut to 3 µm, and stained with hematoxylin and eosin (H&E). Histologic inflammation was evaluated by a board-certified pathologist, using an established histologic scoring system for colitis. Each of five components (degree of ulceration, re-epithelialization, active inflammation, chronic inflammation, and transmural inflammation) was scored and added to calculate the total inflammatory score (TIS). The pathologist was blinded to mouse genotype and treatment group. Images were acquired using an Olympus VS120 slide scanner equipped with a 10× objective and 2/3″ high sensitivity/high resolution CCD camera (Olympus Life Science, Waltham MA).

Apoptosis assays

Formalin-fixed, paraffin-embedded colon sections (~5 µm thickness) were deparaffinized and rehydrated using sequential incubations in xylene, graded series of ethanol, and double-distilled water (5 min each). Tissue sections were permeabilized using freshly prepared 0.1% Triton-X100 with 0.1% sodium citrate, for 10 min at room temperature. Slides were rinsed twice with PBS, then labeled with TUNEL (Terminal deoxynucleotidyl transferase dUTP nick end labeling) stain, according to the manufacturer's instructions (in situ cell death detection kit, fluorescein, Roche). Slides were washed twice after incubation and mounted with mounting media with DAPI (Vectashield, Vector Laboratories) and imaged using a fluorescent microscope (Biotek Cytation 5).

Murine bone marrow transplantation

Recipient mice were lethally irradiated (1000 rad) on the morning of transplantation. Bone marrow (BM) was harvested from femurs and tibias of 8- 12-week-old WT or Sam68 KO mice (mixed male and female) with RPMI (10% FBS), and cell suspensions were washed and diluted to a concentration of 30×10^6 cells/ml in HBSS. 7.5×10^6 cells were injected retro-orbitally into each recipient mouse. Following BMT, all mice were placed on antibiotic water (containing 0.7 mM neomycin sulfate, 80 mM sulfamethoxazole, and 0.37 mM trimethoprim) for 2 weeks before being switched to standard autoclaved water to reconstitute normal gut flora. BMT mice were treated with DSS eight weeks following transplant.

Primary intestinal organoids

Colonic epithelial cells [20] were isolated from adult (8- to 12-week-old) WT or Sam68 KO mice using EDTA (2.5 mM EDTA in HBSS/FBS, 300 rpm for 15 min at 37 °C). Cells were washed, strained, and crypts were counted. Crypts were suspended in IntestiCult Organoid Growth Medium (StemCell Technologies) at a concentration of 10^6 crypts/ml. 30 µl of crypt suspension was mixed 1:1 with Matrigel basement membrane preparation

(Corning) and seeded onto tissue culture dishes. Following 30 min at 37 °C, Matrigel domes were covered with IntestiCult media. Intestinal organoids were visible by day 4–5 of culture, and used for experiments on day 9–12 of culture.

RNA extraction and qPCR analysis

Total RNA was isolated from primary cells and organoids using the High Pure RNA Isolation Kit (Roche Life Science, Indianapolis, IN) or DNAaway RNA miniprep kit (Bio Basic), and from tissue samples using Trizol-based extraction. Reverse-transcription was performed using High Capacity Reverse Transcription kit (Applied Biosystems, Cambridge). Real-time quantitative PCR was performed using SYBR green reagents on a Bio-Rad real-time PCR system. The details of the qPCR in compliance with the recommendations provided at www.rdml.org/miqe.html were as follows: Program—Step 1: 95 °C, 3 min, Step 2: 95 °C, 4 s, Step 3: 60 °C, 60 s, read, then go to step 2 × 40 times. Master Mix: Power SYBR Green or PowerUP SYBR Green Master Mix (Thermo Fisher Scientific); polymerase: Dual-Lock Taq DNA Polymerase (proprietary version of Taq polymerase); $MgCl_2$ final concentration: 2.5 mM; dye: SYBR Green I; Fwd/Rev PRIMER final concentration: 500 nM; machine used: CFX96 (Bio-Rad); reaction volume: 10 µL; consumables used: HARD-SHELL PCR plate, 96-well, thin wall (Catalog #: HSP9601, Bio-Rad); transparency: clear; and sealing method: adhesive (Catalog #: 236366, Thermo Fisher Scientific). Experimental triplicate samples were run for at least three biological replicates for all stimulation conditions. All values were normalized to the housekeeping gene L32. Data are presented as mean ± standard error of mean (SEM) ($n = 3$). p values were obtained by unpaired Student's t test; $^{****}p < 0.0001$, $^{***}p < 0.001$, $^{**}p < 0.01$, $^{*}p < 0.05$.

Western blotting

Total cell lysates were prepared by suspending the cells in the lysis buffer (1.0% Triton-X100, 20 mM HEPES (pH 7.6), 0.1% SDS, 0.5% Sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, and complete protease inhibitor cocktail) and rotating at 4 °C for 30 min. Cytoplasmic and nuclear extracts were prepared as described previously [21]. For Western blot analysis, cell lysates were run through 9% SDS–PAGE gels, transferred onto nitrocellulose membranes, probed using relevant antibodies, and visualized by enhanced chemiluminescence assay.

Data analysis and statistics

Data analysis was performed using GraphPad Prism 7 (GraphPad Software, San Diego, CA). All data are presented as mean ± SEM unless otherwise noted. For direct comparison of two groups, statistical significance was assessed using two-tailed unpaired Student's t tests. Statistical significance of experiments containing multiple groups were assessed using one-way analysis of variance (ANOVA) with Bonferroni's corrections. p values < 0.05 were considered significant.

Results

Sam68 KO mice exhibit protection from experimental, DSS-induced colitis

Our previous work identified novel roles for Sam68 in both TNF/TNFR1 [12] and TLR2/3/4 signaling [13]. In particular, we found that Sam68 is required for NF-κB inflammatory signaling in response to TNF/TNFR1 engagement, as well as for formation of active caspase-8 containing apoptosome and TNF-induced apoptosis. Given the central role of TNF signaling in inflammatory bowel disease (IBD) and the mixed success of blocking TNF in IBD patients, we reasoned that inhibition of Sam68 may be an effective strategy to dampen experimental IBD in vivo.

To test this, we subjected Sam68 KO mice and wild-type (WT) littermate controls to a standard model of acute intestinal damage, wherein mice are fed for 5 days with 3% dextran sulfate sodium (DSS)-supplemented water and killed on day 6 [25]. As expected, both WT and Sam68 KO mice exhibited signs of experimental colitis in response to DSS feeding, including weight loss and colon shortening. However, Sam68 KO mice consistently developed less severe disease compared to WT mice: whereas WT mice lost an average of 15% body weight over 5 days of DSS feeding (Fig. 1A, solid blue line), Sam68 KO mice lost an average of only 8.5% body weight (Fig. 1A, solid red line). Vehicle-treated mice maintained a consistent body weight, as expected (Fig. 1A, dashed lines).

DSS-mediated epithelial damage results in loss of tissue architecture and eventual fibrosis, resulting in thinning and shortening of the colon tissue [26]. DSS-treated Sam68 KO mice showed mild colon shortening (average of 11.2% reduction in colon length compared to vehicle-treated Sam68 KO mice), whereas DSS-treated WT mice showed more severe colon shorting (average of 27.7% reduction in colon length compared to vehicle-treated WT mice) (Fig. 1B, C). The colon lengths of baseline Sam68-KO mice are slightly shorter than those of baseline WT mice (average length: 7.0 cm for Sam68-KO, compared to avg. 7.5 cm for

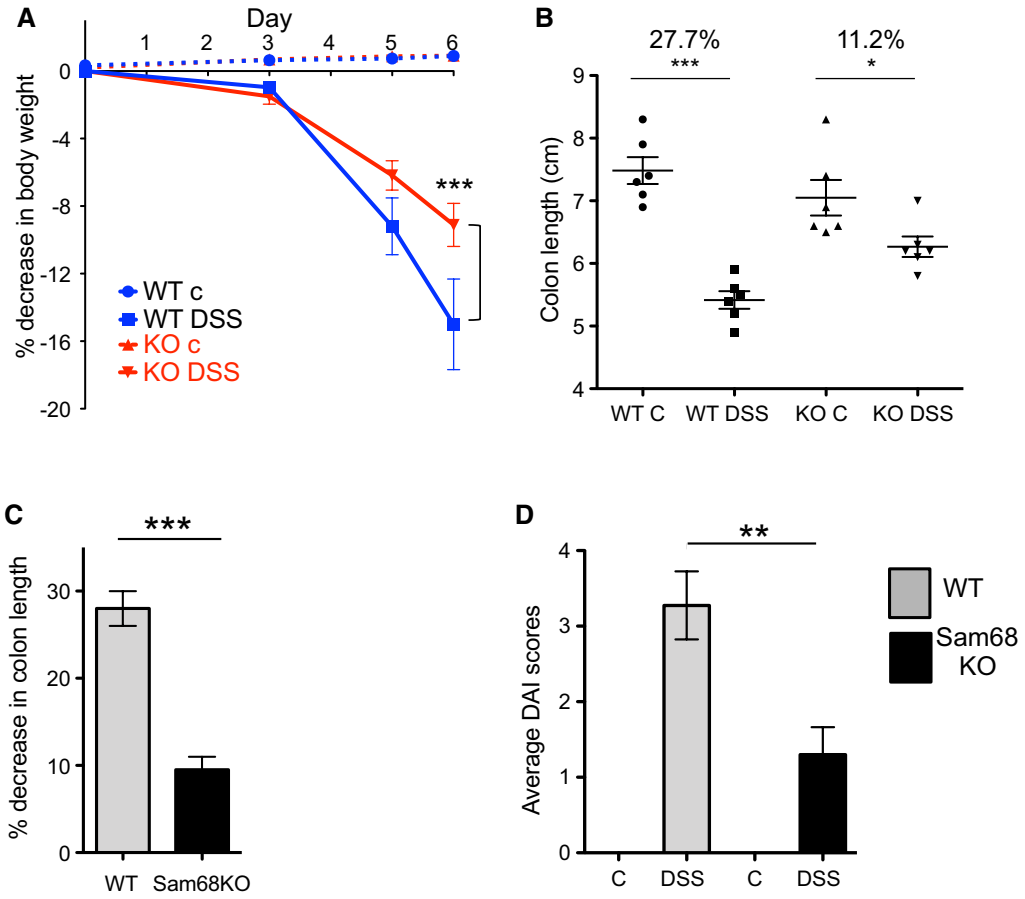

Fig. 1 Sam68 KO mice exhibit resistance to acute DSS-induced colitis. WT and Sam68 KO littermate mice (9–12 weeks old) were supplemented with 3% DSS in their drinking water for 5 days ("DSS") or received regular tap water as controls ("C"). **A** Body weight change during DSS treatment; comparison of WT-DSS group and Sam68 KO-DSS group, two-tailed unpaired t test, $n=6$ mice per group. **B** Colon length in centimeters. Comparison of WT-C vs WT-DSS (average decrease 27.7%) and Sam68 KO vs Sam68 KO-DSS (average decrease 11.2%). Direct comparison of two groups using two-tailed unpaired t test, $n=6$ mice per group. **C** Quantitation of colon shortening in B. % decrease in colon length compared to non-DSS treated controls, $n=6$. **D** Disease activity index (DAI), for indicated mice following DSS treatment or control. Direct comparison of two groups (WT-DSS vs Sam68 KO-DSS) using two-tailed unpaired t test, $n=6$). WT-C and Sam68 KO-C were excluded from the statistical analysis as their DAI scores were zero. All data represented as mean ± SEM. $*p<0.05$, $**p<0.01$, $***p<0.001$

WT controls), likely due to the overall smaller size of the Sam68KO mice [27].

A standard assessment of disease activity (disease activity index, DAI [28, 29]) was performed as described previously [25] on WT and Sam68 KO mice following 5 days of DSS or vehicle control feeding. In agreement with weight loss and colon shortening data (Fig. 1A–C), DAI scores were significantly higher for WT mice (Fig. 1D, gray bars) compared to Sam68 KO mice (Fig. 1D, black bars). Representative images of DSS treated WT and Sam68 KO mice as well as their stools are shown in Supplementary Fig.S1A.

To study the microscopic changes associated with experimental colitis, histopathological assessment of H&E-stained colon tissues from DSS- versus control-fed mice was performed. Colons isolated from control-fed WT and Sam68 KO mice appeared histologically and morphologically normal, with characteristic architecture including columnar epithelia overlying the mucosa, submucosa, and muscularis layers (Fig. 2A, left). This suggests that absence of Sam68 does not affect basal intestinal architecture. In contrast, colon tissues isolated from DSS-treated mice exhibited significant loss of healthy colonic architecture, including epithelial erosion, ulceration, and depletion of mucous-producing goblet cells (Fig. 2A, right). These features were significantly more pronounced in WT mice compared to Sam68 KO littermates (Fig. 2A). Colon tissues isolated from DSS-treated WT mice also exhibited significantly higher proportions of immune cell infiltration (Fig. 2A) including $CD3^+$ T cells (Fig. 2B), compared to DSS-treated Sam68 KO mice.

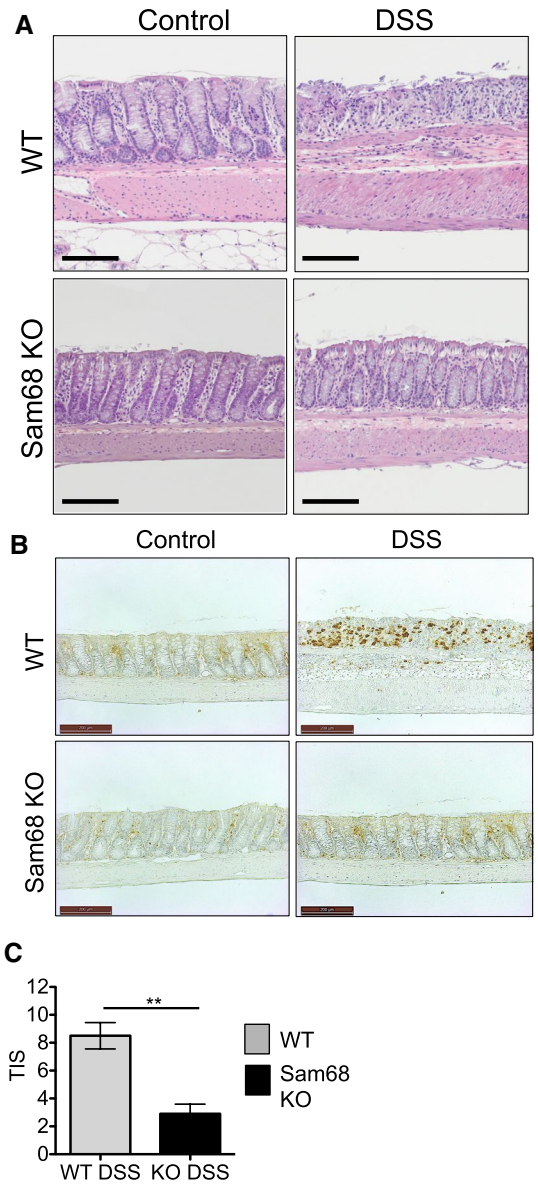

Fig. 2 Sam68 KO mice exhibit reduced colonic inflammation in response to DSS. WT and Sam68 KO littermate mice were fed 3% DSS for 5 days. Representative images of **A** H&E-stained and **B** anti-CD3-stained distal colon sections are shown (scale bars 200 µm). **C** Total inflammatory score (TIS). Direct comparison of two groups, WT-DSS vs Sam68 KO-DSS, using two-tailed unpaired *t* test, $n=6$. Data is expressed as mean ± SEM. $^{**}p<0.01$

To quantitate the inflammatory changes associated with DSS feeding in WT versus Sam68 KO mice, total inflammatory scoring (TIS) was performed on H&E-stained colon tissues using an established scoring system [30]. Sam68 KO exhibited significantly lower TIS compared to WT controls (Fig. 2C), indicating that DSS-induced colonic inflammation is less severe in the absence of Sam68.

Sam68 KO mice are resistant to DSS-induced upregulation of proinflammatory cytokines and apoptosis

Sam68 enhances TNF mediated inflammatory signaling cascades via activation of NF-κB, which is a key regulator of inflammatory gene expression [12, 13]. To better understand the pathogenic mechanisms of Sam68 in acute intestinal inflammation, we assessed NF-κB-dependent proinflammatory gene expression in full-thickness colon tissues isolated from DSS- versus control-fed mice. As expected, the expression of several NF-κB-dependent inflammatory genes relevant to human and murine colitis—including *Tnf*, *Ccr2*, *Csf2*, *Il33*, and *Cxcl10* (encoding TNFα, C–C Motif Chemokine Receptor 2, GM-CSF, IL-33, and IP-10, respectively) was significantly upregulated in colon tissues obtained from DSS-treated, WT mice compared to control-treated mice (Fig. 3A, gray bars). In contrast, colon tissues obtained from DSS-treated, Sam68 KO mice showed very modest changes in gene expression (Fig. 3A, black bars), indicating that Sam68 contributes to the upregulation of these core proinflammatory genes.

Because Sam68 is known to regulate the expression of apoptotic genes downstream of NF-κB [12, 17], we assessed the induction of apoptosis in distal colon tissues isolated from DSS-treated mice versus controls. As expected, DSS treatment of WT mice resulted in substantial induction of apoptosis, as assessed by fluorescent TUNEL (Terminal deoxynucleotidyl transferase dUTP nick end labeling) staining (fluorescein staining, Fig. 3B, left). However, Sam68 KO mice treated with DSS exhibited only moderate TUNEL-positive staining (fluorescein staining, Fig. 3B, right), indicating that DSS-induced apoptosis is reduced in the absence of Sam68.

Expression of Sam68 in hematopoietic cells does not significantly contribute to intestinal inflammation

Sam68 is broadly expressed by a wide variety of cell types, including hematopoietic cells such as lymphocytes [14, 22, 31] and myeloid cells [13, 32], and non-hematopoietic cells, such as colonic epithelial cells and fibroblasts [12, 22]. We utilized bone marrow transplant (BMT) experiments to determine the cellular source of proinflammatory Sam68 in the intestine in response to DSS. Briefly, WT recipient mice were lethally irradiated and then re-constituted with bone marrow isolated from either WT or Sam68 KO donor mice (WT → WT and KO → WT, respectively). Resulting chimeric mice were subjected to acute DSS feeding, as described above, and sacrificed on day 6. Interestingly, WT → WT and KO → WT BMT cohorts exhibited comparable responses to DSS, in terms of weight loss (Fig. 4A), DAI

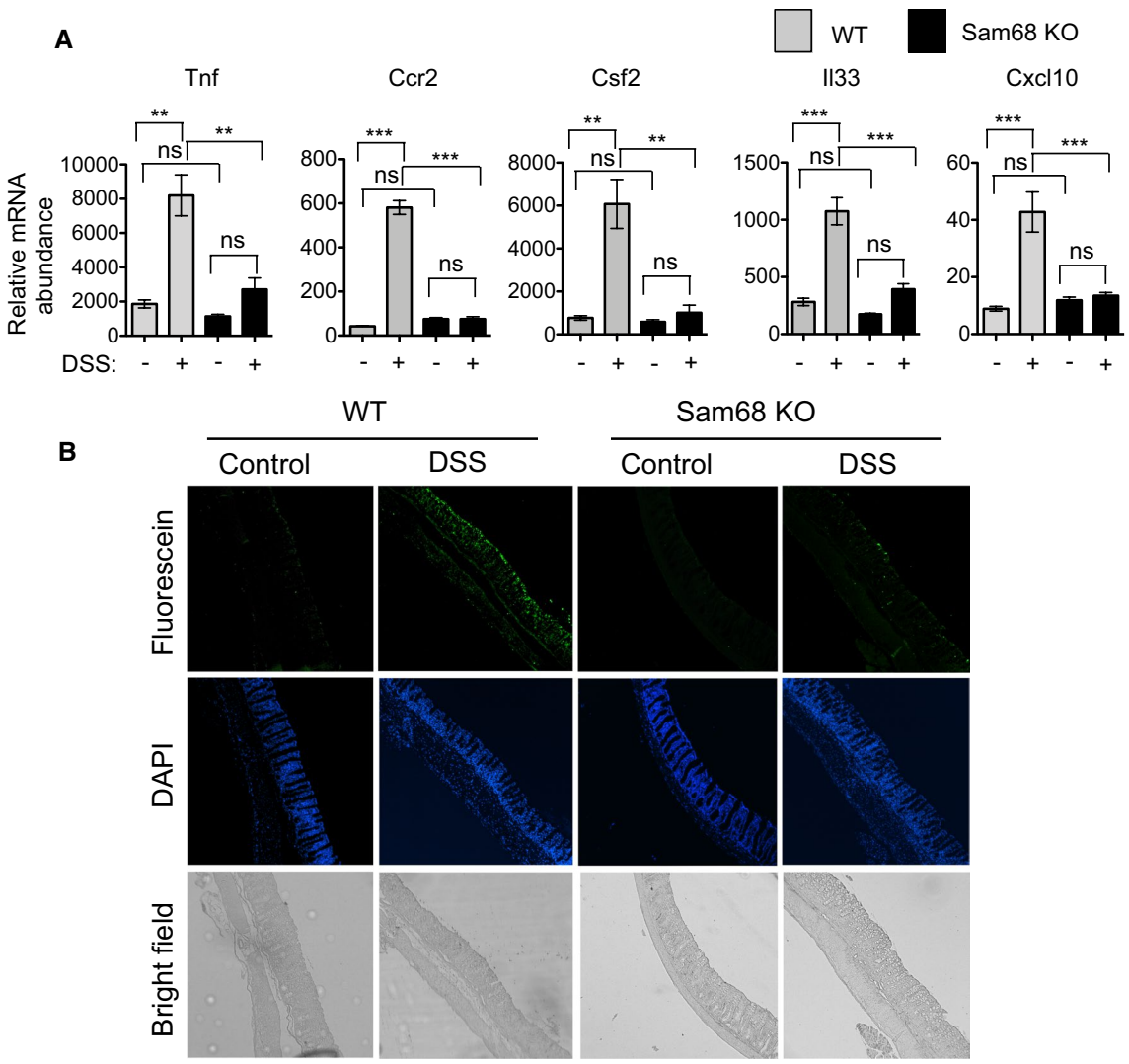

Fig. 3 DSS-induced proinflammatory gene expression and apoptosis are decreased in the colon of Sam68 KO mice. WT and Sam68 KO littermate mice were fed 3% DSS for 5 days. **A** RNA was isolated from colon tissues on day 6 and relative gene expression was normalized to that of the ribosomal housekeeping gene L32. Data represented as mean ± SEM (one-way ANOVA, Bonferroni's multiple comparisons test, $n =$). $^{**}p < 0.01$, $^{***}p < 0.001$. (B) Distal colon sections were assessed for apoptosis by fluorescent TUNEL staining (upper row). Nuclear staining (DAPI, middle row) and bright field (bottom row) are also shown

(Fig. 4B), colon shortening (Fig. 4C), and histopathological TIS (Fig. 4D), and absence of Sam68 in hematopoietic cells offered no protection against DSS-induced intestinal damage. We confirmed that bone marrow transplantation efficiency was comparable in the two groups of mice, i.e., WT → WT and KO → WT, by flow cytometry-based analysis of T cells, Treg cells, B cells and myeloid cells in the spleen of recipient mice (Supplementary Fig. S2).

The lack of protection among KO → WT BMT mice indicates that hematopoietic cell expression of Sam68 does not significantly contribute to intestinal inflammation. Consistent with this, the frequency of major lymphoid and myeloid populations, including B and T cells, NK cells, macrophages, and dendritic cells, was unchanged in the spleen (Supplementary Fig. S3A), thymus (Supplementary Fig. S3B) and mesenteric lymph nodes (Supplementary Fig. S4) of Sam68 KO mice compared to WT controls, further suggesting that Sam68 does not play a major role in hematopoietic cell development, differentiation or trafficking.

TNF-induced proinflammatory gene expression is compromised in Sam68 KO three-dimensional intestinal organoids

Our observations in BMT chimeric mice led us to hypothesize that non-hematopoietic Sam68, potentially in the IEC compartment [18], is a major contributor to DSS-induced intestinal damage. Primary IECs are prone to

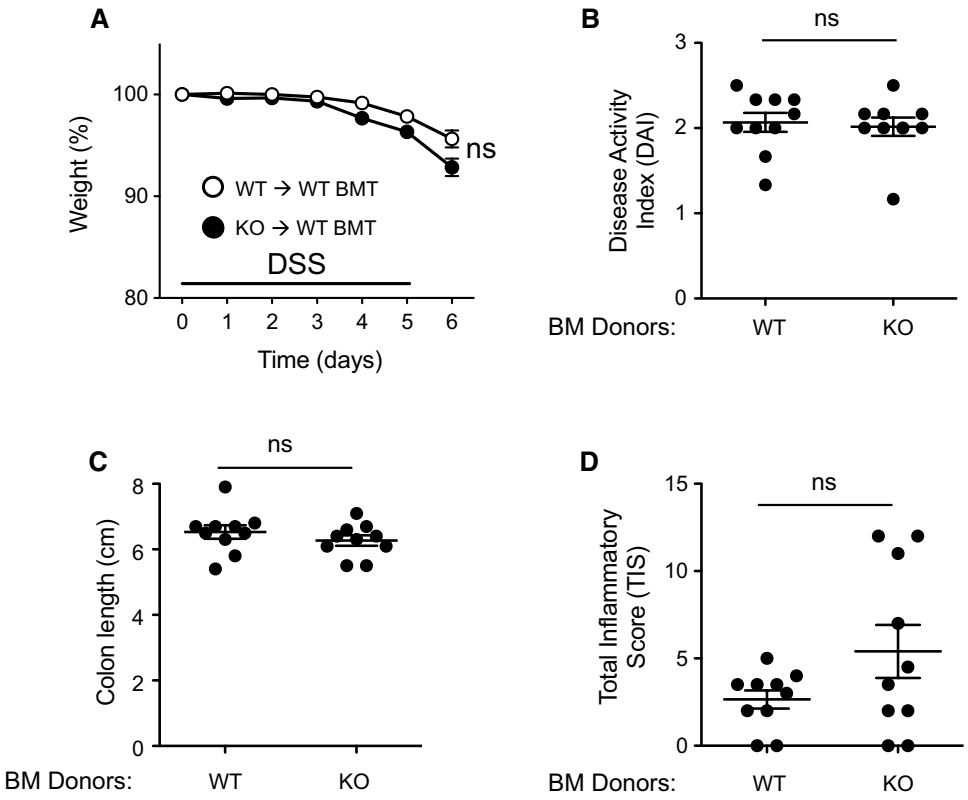

Fig. 4 Sam68 expression in hematopoietic cells does not contribute significantly to acute DSS-induced colitis. 8- to 10-week-old WT mice (mixed M/F) were lethally irradiated (1000 rad) and reconstituted with bone marrow (BM) cells from WT or Sam68 KO mice ($8–10 \times 10^6$ cells/recipient). 8 weeks post-transfer, mice were treated for 5 days with 3% DSS-supplemented drinking water. All mice were sacrificed on day 6. **A** Body weight change over time. **B** Disease activity index (DAI), based on weight loss, stool consistency, and fecal blood contents at day 6. **C** Colon length. **D** Total inflammatory colitis score (TIS), based on histopathological evaluation of H&E-stained colon tissues. Direct comparison of two groups using two-tailed unpaired t test. All data represent mean ± SEM ($n = 10$ recipient mice/group; ns not significant)

rapid apoptosis once removed from the underlying mucosa [33] and do not fully recapitulate the complex spatial organization of the intestinal epithelium. Therefore, we developed a three-dimensional organoid system in which to study Sam68 KO IECs ex vivo. Following 8–10 days of culture, WT and Sam68 KO intestinal organoids were fully differentiated, modeling the self-renewal capacity and differentiation hierarchy found in the primary tissue, including epithelial polarization and functional lumen (Fig. 5A).

To investigate how Sam68-deficient colonoids respond to physiologically relevant proinflammatory signaling, we treated WT and Sam68 KO colonoids with TNF (100 ng/ml) ex vivo for 3 h, and then lysed the colonoids for mRNA and qPCR. Interestingly, and in agreement with our in vivo data (Figs. 1, 2, 3, 4), we observed significant enhancement in proinflammatory gene expression (*Tnf*, *Ccr2*, *Csf2*, and *Cxcl10*) among WT colonoids in response to TNF stimulation (Fig. 5B, gray bars). In contrast, Sam68-KO colonoids treated with TNF showed less-efficient induction of *Tnf* and *Cxcl10*, and no induction of *Ccr2* or *Csf2* (Fig. 5B, black bars). Gene expression of *Il33* was not induced by TNF treatment of either WT or Sam68-KO colonoids (Fig. 5B), suggesting that its induction in DSS-treated colon tissues (Fig. 3A) may depend on cell type(s) not present in colonoids, such as mucosal immune cells, or may occur at different kinetics than 3 h in colonoids.

Sam68 is selectively upregulated in inflamed regions of UC patient colon tissues

UC is responsive to TNF-directed therapeutics [35–38], suggesting that TNF is a major inflammatory driver of disease. To more closely examine the association of Sam68 with active inflammation in UC, we obtained colonic biopsy samples from actively inflamed and non-inflamed regions of the colon of UC patients, and non-IBD controls, and prepared mRNA and protein extracts. We found that Sam68 protein was dramatically increased in inflamed regions of UC patient colon tissue compared to non-inflamed UC colon tissues and healthy controls (Fig. 6A). This suggests that Sam68 expression correlates with active inflammatory signaling.

Interestingly, the enhanced Sam68 expression appears to be regulated by a posttranslational mechanism, as we did not find any significant differences in the mRNA expression of Sam68 between the control and UC samples (Fig. 6B). However, mRNA transcript levels of several proinflammatory genes were increased in UC patient biopsy samples (including *TNF, CCR2, CSF2, IL-33, and CXCL10*, Fig. 6C), suggesting active proinflammatory transcription in UC correlating with increased Sam68 protein expression (Fig. 6A). These data suggest the intriguing possibility that UC patients may exhibit differential posttranslational

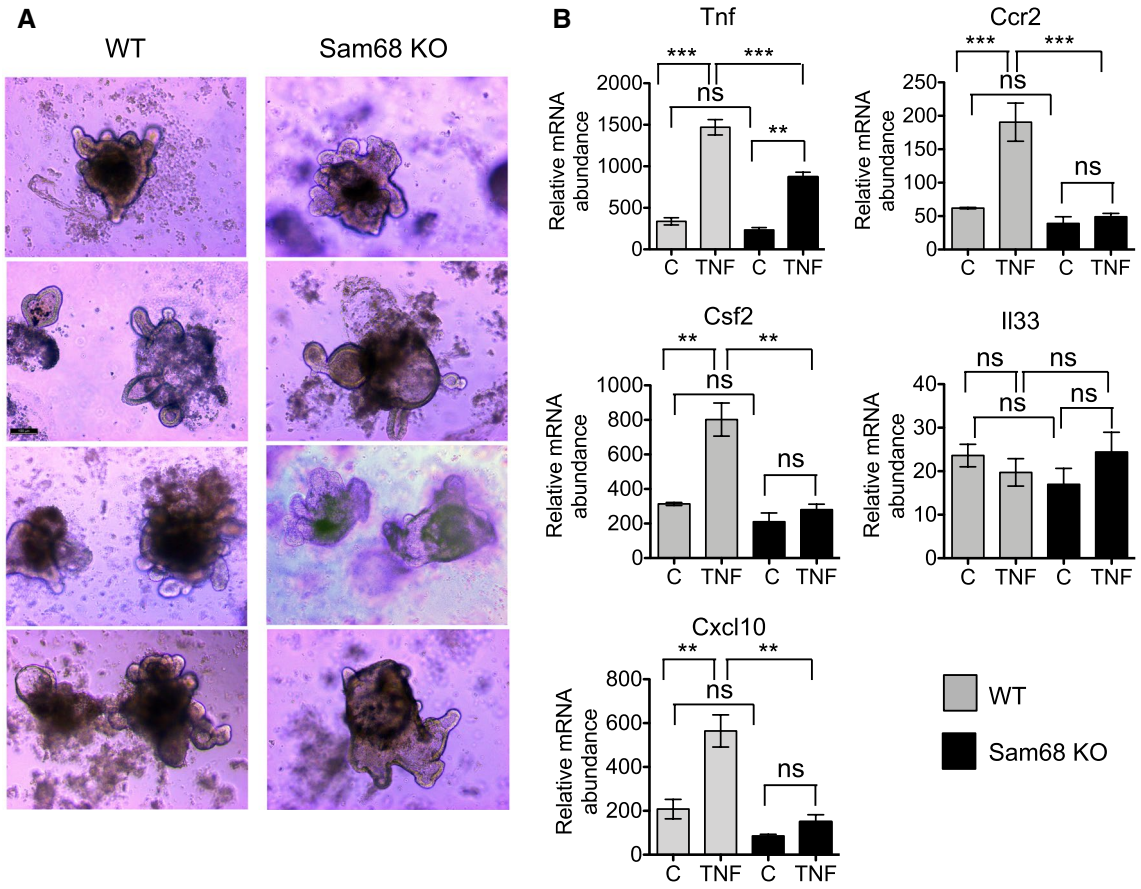

Fig. 5 Sam68 KO organoids show decreased TNF induced inflammatory gene expression. **A** Representative images of colon organoid cultures prepared from WT and Sam68 KO mice ($n=5$). **B** Organoids were stimulated with TNF for 3 h and samples were then analyzed by qPCR to determine the expression of indicated mRNAs relative to that of the ribosomal protein L32. Relative mRNA expression data were calculated with respect to the expression of ribosomal protein L32. Data represented as mean ± SEM (One way ANOVA, Bonferroni's multiple comparisons test, $n=3$). $^{**}p<0.01$, $^{***}p<0.001$, *ns* not significant

stabilization of Sam68, resulting in overexpression of the protein and chronic inflammatory signaling.

To further examine whether proinflammatory signaling enhances Sam68 protein levels, we stimulated human colon cells (HT-29 cell line) and mouse embryonic fibroblasts (MEF cells) with TNF. We found that Sam68 protein levels were substantially elevated in both cell types following TNF treatment (Fig. 6D). To directly study the mechanism involving Sam68 in TNF-induced proinflammatory signaling in the colon, we generated a Sam68-deficient HT-29 cell line by CRISPR/Cas9-mediated gene editing as we described previously [39] (Fig. 6E). We found that the absence of Sam68 blocked TNF-induced degradation of IκBα protein in the cytoplasm (Fig. 6E top), with associated inhibition of the nuclear translocation of NF-κB subunits, RelA, p50 and c-Rel (Fig. 6E bottom).

Collectively, protection of Sam68 KO mice from experimental colitis enhanced the expression of Sam68 protein in UC patients' colon tissues, induction of Sam68 following TNF stimulation, and inhibition of TNF-induced activation of NF-κB pathway in colon cells lacking Sam68 suggest a proinflammatory role for Sam68 in perpetuating chronic inflammation of the colon (Fig. 7 schematic model).

Discussion

Ulcerative colitis (UC) is an idiopathic form of inflammatory bowel disease (IBD) characterized by continuous, chronic inflammation of the colonic mucosa [34]. Unchecked inflammation is highly destructive, leading to permanent tissue damage and organ-specific autoimmunity [40]. Thus, an improved understanding of the molecular events regulating inflammatory signaling pathways is critical for the control of inflammatory diseases such as UC. Although UC is associated with Th2-like cytokine production, the expression of T cell-derived cytokines including IL-4 and IL-5 is variable among patient samples [41, 42]. In contrast, inflamed tissues

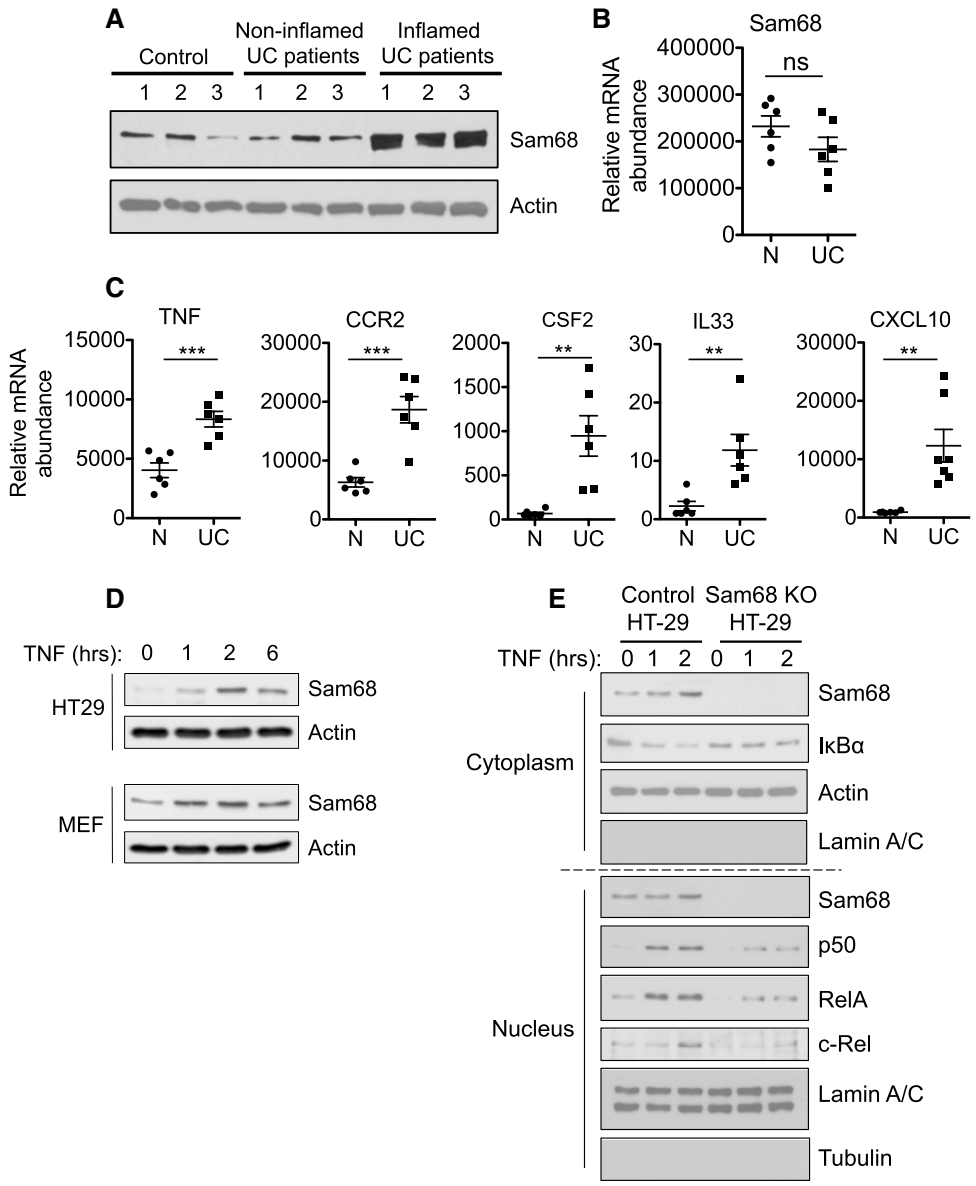

Fig. 6 UC and TNF stimulation enhances Sam68 protein and inflammatory gene expression and Sam68 deficiency blocks TNF-induced NF-κB pathway in colon cells. **A** Protein expression of Sam68 and actin in colonic biopsy samples isolated from indicated patient groups ($n=3$/group). **B** and **C** Relative mRNA expression of indicated genes among colonic biopsy samples isolated from healthy control ("N") and inflamed ulcerative colitis (UC) patients. Relative mRNA expression data were calculated with respect to the expression of ribosomal protein L32. Direct comparison of two groups, (N and UC) using two-tailed unpaired t test. Data represented as mean ± SEM. $^{**}p<0.01$. $^{***}p<0.001$. **D** HT-29 and MEF cells were treated with 100 ng/ml TNF for the indicated time points ($n=3$). Total cell lysates were probed for Sam68 expression. Actin was used as loading control. **E** HT-29 cells were infected with control or Sam68 guide RNA and Cas9 expressing lentiviral vectors. Transduced cells were selected using puromycin and analyzed for Sam68 expression and TNF-induced NF-κB pathway activation. Cells were treated with TNF as indicated and analyzed for the expression of indicated proteins. Actin and Lamin A/C were used as loading controls for the cytoplasm and nucleus, respectively. Purity of the lysate fractions was confirmed by probing for Lamin A/C in the cytoplasm and tubulin in the nucleus ($n=3$)

from UC patients consistently express overactivation of TNF signaling pathways [6, 8], suggesting that these are key drivers of inflammation in UC. Therapeutic agents targeting TNF, including infliximab, adalimumab, and golimumab, are mainstays for the treatment of patients with moderate-to-severe UC [35–38], highlighting the central role of TNF in UC pathogenesis. Although these agents have resulted in significant clinical improvements for a large proportion of patients, many patients fail to express durable remission, or develop unwanted—and often severe—side effects [43, 44]. Thus, there is a clear need for improved therapeutic options for UC patients.

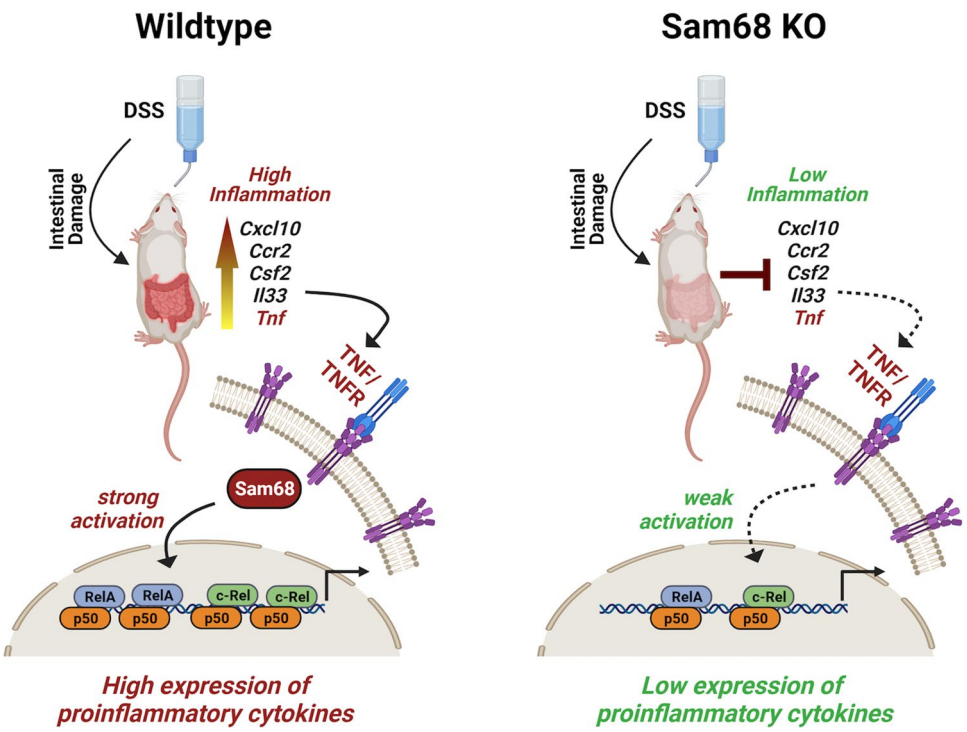

Fig. 7 A schematic model showing the role of Sam68 in intestinal inflammation. One possible mechanism involving Sam68 in intestinal inflammation is the activation of the canonical NF-κB pathway through the induction of RelA/p50 and c-Rel/p50 dimers. These canonical NF-κB dimers control the expression of several proinflammatory genes, including *Tnf*, *Ccr2*, *Csf2*, *Il33*, and *Cxcl10*. DSS treatment enhances the expression of these proinflammatory genes and the TNF produced further activates the inflammation in the presence of Sam68 (left). In the absence of Sam68, DSS-induced inflammation is greatly diminished, and TNF-induced NF-κB RelA/p50 and c-Rel/p50 activation is compromised in colon cells. This decreased inflammatory signaling might play a role in protecting the Sam68 KO mice from experimental colitis (right), which may be translatable as an approach to treat the human disease through targeting Sam68

In addition to its canonical roles in RNA metabolism, Sam68 functions as an adaptor protein that contributes to the regulation of intracellular signaling cascades downstream of T cell receptor signaling [14, 22] and insulin signaling [23, 24], among others. Our previous work identified Sam68 as a critical component of downstream signaling in response to TNF receptor activation [12], placing it at the nexus point of IBD-relevant proinflammatory signaling pathway.

Based on our findings, we hypothesized that Sam68 contributes to chronic inflammation in UC, and that it may represent an attractive target for future drug design because of its critical role in TNF signaling. In this report, we show that Sam68 protein is significantly upregulated in inflamed mucosal biopsy samples from UC patients compared to non-inflamed UC samples and healthy control colon samples. In agreement with a previous report implicating Sam68 in UC [18], we found that Sam68 over-expression in UC patient tissues was restricted to actively inflamed regions of the colon. This finding is significant because it implicates Sam68 in destructive tissue inflammation. Interestingly, although we found that mRNA transcript levels of several proinflammatory genes were increased in UC patient samples compared to non-inflamed controls, Sam68 gene expression was comparable among controls and UC patients. Collectively, these findings suggest that overexpression of Sam68 protein in UC patients' colon tissues may enhance TNFR-dependent activation of NF-κB [12], thus perpetuating chronic inflammation. Our data also suggest that posttranslational modifications may stabilize Sam68 in UC patients, resulting in overexpression of the protein [18].

Previous studies have shown tyrosine phosphorylation of Sam68 [45, 46], and threonine/serine phosphorylation [47] as well as phosphorylation by other Src family kinases. In our previous study [12], we found that TNF stimulation does not induce tyrosine phosphorylation of Sam68. Whether TNF induces serine/threonine phosphorylation of Sam68, which may alter protein stability remains to be determined. Defective ubiquitin-dependent proteasomal degradation could be a mechanism stabilizing Sam68 in UC and inflammatory conditions. Posttranslational modifications such as acetylation and methylation alters proteasomal susceptibility of proteins; e.g., acetylation of Smad7 prevents its proteasomal degradation in IBD [48] and arginine methylation has been shown to stabilize KLF4 [49, 50]. Previous studies have shown that Sam68 is modified by acetylation [51] and PRMT1-mediates asymmetric dimethylation of Sam68 [52].

Whether acetylation and methylation play a role in stabilizing Sam68 warrants further investigation.

Our present study is the first to examine the in vivo effects of an intestinal damage model (DSS colitis) in Sam68 KO mice. Deletion of Sam68 contributes to reduced body size [27], and our Sam68 KO mice also appeared smaller than the wild-type mice; however histological colon tissues appeared relatively normal among our Sam68-KO mice (Fig. 2, Supplementary Fig. S1). Upon challenge with an acute protocol of DSS, however, we observed significantly less colonic inflammation, tissue damage, and apoptosis among Sam68 KO mice compared to WT littermate controls. These data suggest that Sam68 contributes to the epithelial damage response to DSS feeding, and that overexpression of Sam68 protein in UC patients may enhance local inflammation at the level of intestinal epithelial cell (IEC) dysregulation.

Previous work suggested that Sam68 contributes to IEC apoptosis via constitutive activation of NF-κB signaling [18]. Our previous studies demonstrated a requirement for Sam68 as an adaptor protein at the TNF receptor mediating TNF-induced NF-κB activation and inflammatory gene expression [12]. In the absence of Sam68, TNF induced activation of the NF-κB pathway is compromised due to the decreased phosphorylation of the kinases at the TNF receptor, which could account for the decreased proinflammatory gene expression in Sam68 KO cells. As several previous studies [53–55] and this study show, DSS induces TNF expression, which could initiate the inflammatory signaling cascade through Sam68-dependent pathway. In the absence of Sam68, both DSS-induced TNF expression and the TNF-induced inflammation are decreased, which may account, at least in part, for the decreased inflammation in Sam68 KO mice.

This constituted the base for our hypothesis that IEC-specific Sam68 actively contributes to DSS-induced colonic inflammation in mice via enhancing TNF-dependent NF-κB pathway activation. In our bone marrow transfer (BMT) experiment in which Sam68 KO or WT bone marrow was transferred to lethally irradiated, WT recipients, we observed no rescue of the colitis phenotype among recipients of Sam68 KO versus WT bone marrow, suggesting that non-hematopoietic (i.e., IEC) Sam68, but not hematopoietic Sam68, may drive colonic inflammation. Since Sam68 KO mice are infertile and newborn Sam68 KO exhibit high rates of neonatal mortality [56, 57], our plan to perform reciprocal BMT using Sam68 KO as recipients of WT versus Sam68 KO BM proved unfeasible. Our immediate future studies are aimed at generating and utilizing novel IEC-specific Sam68 conditional KO mice, which will further validate the epithelial specific role of Sam68 in intestinal inflammation.

To gain additional insight into the role of Sam68 on the intestinal epithelium ex vivo, we developed three-dimensional colonic organoid cultures from primary crypt cells of WT and Sam68 KO mice. Colonic crypts isolated from both WT and Sam68 KO mice yielded organoids in culture, and the lack of Sam68 resulted in a significant decrease in the expression of selected TNF-induced inflammatory genes (*Tnf*, *Ccr2*, *Csf2*, and *Cxcl10*) in the 3D culture. Providing mechanistic evidence, we found that CRISPR/Cas9 mediated deletion of Sam68 in a colon cell line (HT-29) blocked TNF-induced NF-κB pathway activation. This suggests that the primary impact of Sam68 in colon inflammation is likely at the level of NF-κB-dependent inflammatory and anti-apoptotic signaling, as we also observed reductions in DSS-induced epithelial apoptosis in Sam68 KO mice. Collectively, these data suggest that Sam68 functions at multiple levels, i.e., proinflammatory signaling and cell survival/death signaling, to regulate inflammation.

Chronic inflammation of the colon and rectum, as occurs in UC patients, is an established risk factor for the development of colorectal cancer (CRC) [58, 59], and Sam68 promotes colon tumorigenesis via regulation of DNA damage-induced NF-κB signaling [17]. Despite routine endoscopic surveillance for CRC among UC patients, this cohort is still at significantly increased risk of developing and dying from CRC [60–62]. Therefore, the identification of key factor(s) contributing to chronic inflammatory signaling in the UC colon is of tremendous importance for ultimately preventing malignant transformation. Our data suggest that Sam68 may represent such a factor, as it is overexpressed selectively in the inflamed regions of colonic tissue from patients with active UC. In support of this hypothesis, expression of Sam68 has been previously correlated with enhanced proliferation and survival of prostate and ovarian cancer cells [16, 63].

Collectively, our present findings and previous study [12] identify Sam68 as a key inflammatory driver in UC through enhancement of TNF-dependent signaling. Although TNF-directed therapeutics offer some benefit in UC, studies show that approximately 50% of patients do not respond consistently to TNF blockade, suggesting more complexity to the role of TNFR signaling in UC. Mechanism(s) underlying the efficacy of anti-TNF agents in some patients, versus failure to respond in others, are not well understood. In addition to its role in inflammation, TNF is critical for multiple biological processes including potential to act as anti-tumor agent through its cytotoxic function and also plays a role in tumor surveillance [64]. Hence, global long-term blocking of its function in IBD patients, in general, may lead to unexpected side effects. Deletion of Sam68, a downstream adaptor in TNFR signaling, greatly reduces TNF-induced expression of various inflammatory mediators, yet other TNF-induced pathways such as ERK and JNK are not affected [12]. Therefore, specifically targeting downstream signaling proteins such as Sam68 involved in TNF-mediated inflammation may

represent a refined therapeutic strategy with potentially lesser side effects, and may reduce the potential for malignant transformation among patients with UC.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s00018-021-03976-7.

Acknowledgements The authors thank Dr. Stephane Richard, McGill University for providing the Sam68 KO mice. We thank the Cleveland Digestive Disease Research Core Center (DDRCC) for support, particularly Drs. Fabio Cominelli, M.D., Ph.D. (Center Director and Mouse Models Core Director), Dr. Theresa Pizarro, Ph.D. (Associate Center Director and Histology/Imaging Core Director), and Dr. Claudio Fiocchi, M.D. (Associate Center Director and Biorepository Core Director). We also thank Dr. Wei Xin, M.D., Ph.D., board-certified DDRCC Pathologist, for his expert analysis of DSS-treated colon tissues.

Author contributions WAG performed experiments, analyzed data, and wrote the manuscript. SB performed experiments and analyzed data. ARL, FDSR and TM performed experiments. PR conceived of the study, performed experiments, analyzed data and edited the manuscript.

Funding This work was supported by supported by the National Institute of Allergy and Infectious Diseases at National Institute of Health grants R01 AI116730 and R21 AI144264, and National Cancer Institute at National Institute of Health grant R21 CA246194, to P.R. Research reported in this publication was also supported by the National Institute Of Diabetes And Digestive And Kidney Diseases at the National Institutes of Health under Award Number P30DK097948 that supported Cleveland Digestive Disease Research Core Center Pilot/Feasibility Award to P.R. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health. W.A.G. was supported by the National Institute Of Diabetes And Digestive And Kidney Diseases at the National Institutes of Health grants R01 DK128143, K01 DK105138, R03 DK123579, and Crohn's & Colitis Foundation Senior Research Award #635911. A.R.L was supported by the National Institute of Health NIH/NEI T32 predoctoral training grant T32EY007157.

Availability of data and material All of the data underlying this article are available in the article and in its online supplementary data. Materials such as reagents will be available upon request.

Code availability Not applicable.

Declarations

Conflict of interest PR has a patent on the use of Sam68 for modulating signaling through the TNF receptor (US8598137B2). Other authors have declared that no conflict of interest exists.

Ethics approval All mouse studies were approved under CWRU IACUC protocol 2013-0134. All human tissue samples used in this study were slated to be discarded samples that were deidentified and procured through the Cleveland Digestive Disease Research Bioreposito Core facility with non-human subject research IRB approval from University Hospitals, IRB number NHR-16-103 and STUDY20210825.

Consent to participate Not applicable. No human subjects were recruited for this study.

Consent for publication Not applicable.

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